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Vaccination of BALB/c Mice with *Escherichia coli*-Expressed Vaccinia Virus Proteins A27L, B5R, and D8L Protects Mice from Lethal Vaccinia Virus Challenge▽†

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The potential threat of smallpox use in a bioterrorist attack has heightened the need to develop an effective smallpox vaccine for immunization of the general public. Vaccination with the current smallpox vaccine, Dryvax, produces protective immunity but may result in adverse reactions for some vaccinees. A subunit vaccine composed of protective vaccinia virus proteins should avoid the complications arising from live-virus vaccination and thus provide a safer alternative smallpox vaccine. In this study, we assessed the protective efficacy and immunogenicity of a multisubunit vaccine composed of the A27L and D8L proteins from the intracellular mature virus (IMV) form and the B5R protein from the extracellular enveloped virus (EEV) form of vaccinia virus. BALB/c mice were immunized with *Escherichia coli*-produced A27L, D8L, and B5R proteins in an adjuvant consisting of monophosphoryl lipid A and trehalose dicorynomycolate or in TiterMax Gold adjuvant. Following immunization, mice were either sacrificed for analysis of immune responses or lethally challenged by intranasal inoculation with vaccinia virus strain Western Reserve. We observed that three immunizations either with A27L, D8L, and B5R or with the A27L and B5R proteins alone induced potent neutralizing antibody responses and provided complete protection against lethal vaccinia virus challenge. Several linear B-cell epitopes within the three proteins were recognized by sera from the immunized mice. In addition, protein-specific cellular responses were detected in spleens of immunized mice by a gamma interferon enzyme-linked immunospot assay using peptides derived from each protein. Our data suggest that a subunit vaccine incorporating bacterially expressed IMV- and EEV-specific proteins can be effective in stimulating anti-vaccinia virus immune responses and providing protection against lethal virus challenge.

Although smallpox has been eliminated from the natural environment due to the massive vaccination program conducted by the World Health Organization in the late 1960s, the potential threat of intentional release of variola virus has renewed the search for a safe, effective smallpox vaccine. The current U.S.-approved vaccine, Dryvax (Wyeth, Marietta, PA), composed of live vaccinia virus, is very effective but has been associated with severe adverse events. These events include postvaccinal encephalitis, myopericarditis, progressive vaccinia, eczema vaccinatum, fetal vaccinia, and, potentially, death (29). Although intentional release of variola virus is of great concern, other orthopoxvirus pathogens occurring in nature, such as monkeypox virus, intermittently infect human populations and pose serious health threats. Because orthopoxviruses are so highly related at the DNA level, it is predicted that any vaccine developed against one poxvirus would stimulate immunity to this entire group of viruses (9, 12, 13).

Vaccinia virus, belonging to genus *Orthopoxvirus*, family *Poxviridae*, is a large (~200-kbp) DNA virus known to produce several distinct infectious virion forms. Immature virions (IV), consisting of progeny DNA molecules, viral enzymes, and structural proteins, are formed immediately after viral DNA replication (32). Although it was previously proposed that the IV is surrounded by a single lipid bilayer that is synthesized de novo (6, 20), it is now thought that the IV acquires a double membrane by budding through the intermediate compartment (between the endoplasmic reticulum and the Golgi compartment) and undergoes proteolytic processing and core condensation to become infectious intracellular mature virus (IMV) (39, 47). After formation, the IMV particles move on microtubules, acquiring a double layer of additional membrane from the early endosomes or *trans*-Golgi network to form the intracellular enveloped virus (IEV) (46). The IEVs then travel to the cell surface along microtubules, where the outermost IEV membrane fuses with the plasma membrane to give rise to cell-associated enveloped virus (34, 46). A portion of the enveloped-virus particles can either remain associated with the cell or be released into the external medium as extracellular enveloped virus (EEV) (4). The EEV form of poxvirus is postulated to be important for long-range dissemination of the virus within the host, while the IMV form is thought to be important for transmission of infection between hosts (4, 45). For this reason, it is thought that a suitable smallpox vaccine must be effective against both infectious forms of this virus (30).

Vaccinia virus encodes more than 200 proteins, several of which have been shown to be important protective antigens

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against poxvirus infections. The vaccinia virus A27L protein is a 14-kDa protein that is found in the envelope of IMV. It is involved in virus-cell attachment, virus-cell fusion, and virus release from cells (5, 40). Antibodies directed against A27L have potent IMV-neutralizing capacity in vitro (27). Intraperitoneal immunization of mice with *Escherichia coli*-expressed A27L protein or passive treatment with A27L-specific monoclonal antibodies provides full protection against lethality in a vaccinia virus challenge model (8, 27, 38), while a DNA vaccine encoding A27L offers partial protection (35). In addition to humoral responses, in vitro restimulation with A27L elicits cellular proliferative immune responses in splenocytes isolated from vaccinia virus-infected mice (8). The D8L protein has also been shown to elicit strong humoral and cellular immune responses in mice (8). This abundant 32-kDa IMV protein plays an important role in virus entry and adsorption due to its ability to bind to chondroitin sulfate on host cells (23, 31). In vitro, antibodies directed against D8L have IMV-neutralizing activity, and soluble D8L protein inhibits the adsorption of wild-type vaccinia virus to cells (23). In vivo, D8L is immunogenic, and immunization of mice with a DNA vaccine encoding D8L provides partial protection from lethal vaccinia virus challenge (35). A recent study by Sakhatskyy et al. has confirmed that D8L induces strong protective antibody responses in vivo and improves the efficacy of polyvalent poxvirus DNA vaccines composed of A27L and B5R or of A27L, B5R, L1R, and A33R (41). Another immunogenic and protective vaccinia virus antigen is the 42-kDa glycosylated EEV membrane protein B5R. Due to its roles in IMV wrapping, EEV morphogenesis, and release of the virus from the cell, B5R is essential for full virulence of vaccinia virus (11, 43, 54). As a result, polyclonal antiserum or monoclonal antibodies directed against B5R are potent inhibitors of EEV infection (2, 26). Mice vaccinated with baculovirus-derived B5R protein are protected against lethal vaccinia virus challenge (16), and vaccination of mice with a DNA vaccine encoding B5R alone is sufficient to protect mice from lethal intranasal challenge (35). B5R also appears to stimulate cellular responses: Pulford et al. have shown that a single dose of a B5R DNA vaccine induced a strong and consistent gamma interferon (IFN-γ) response in BALB/c mice (35).

The importance of including A27L, D8L, and B5R as components of a subunit smallpox vaccine is underscored by the detection of antibody responses to both IMV- and EEV-associated proteins in the sera of humans immunized with Dryvax (25) or vaccinia virus strain Lister (36). Studies with nonhuman primates or mice have also demonstrated that immunization with baculovirus-expressed recombinant proteins or DNA vaccines composed of different combinations of IMV- and EEV-associated proteins—such as A33R, B5R, and L1R (14, 15), A27L, A33R, L1R, and B5R (21), or A27L, B5R, L1R, A33R, and D8L (41)—result in the induction of high levels of neutralizing antibodies and in the protection of mice from lethal challenge with vaccinia virus. In this study, we describe the expression of immunogenic recombinant A27L, D8L, and B5R vaccinia virus proteins by *E. coli* and demonstrate that subcutaneous immunization of BALB/c mice stimulated high levels of protein-specific and virus-cross-reactive immunoglobulin G (IgG) responses. These antibodies exhibited neutralizing activity against vaccinia virus, and immunization with the proteins provided full protection against lethal doses of vaccinia virus delivered via the intranasal route. Passive transfer of serum, but not of total splenocytes, from protein-immunized mice also provided full protection against the lethal vaccinia virus challenge, suggesting that antibodies were the important correlate of protection in this model system.

MATERIALS AND METHODS

Bacterial strains, plasmids, virus, and cell lines. Primers, the pCR2.1 TA cloning vector, and the *E. coli* strain INVα F′ were all purchased from Invitrogen (Carlsbad, CA). The *E. coli* protein expression vector pET41b and *E. coli* strains Rosetta and BL21(DE3) were obtained from Novagen (San Diego, CA). The Western Reserve strain of vaccinia virus (VV-WR) was obtained from the American Type Culture Collection (ATCC), Manassas, VA (VR-119), and was propagated as described elsewhere (22). The Copenhagen and International Health Department-J strains of vaccinia virus (VV-COP and VV-IHD-J, respectively) were obtained from the laboratory of Dennis Hruby (Oregon State University, Corvallis). BSC-40 cells were purchased from the ATCC (CRL-2761) and were propagated according to ATCC protocols.

***E. coli* expression and purification of vaccinia virus A27L, D8LΔ, and B5RΔ proteins.** The GenBank accession numbers for the A27L, D8L, and B5R proteins of VV-COP are P20535, P20508, and P21115, respectively (17). The genes encoding the A27L (amino acids [aa] 2 to 110), D8LΔ (aa 2 to 260), and B5RΔ (aa 22 to 276) proteins were generated by PCR using the following primer pairs, respectively: A27L-Forward (5′-CGGGGTACCGACGGAACTCTTTTCCCC-3′) and A27L-Reverse (5′-CCGGAATTCCTCATATGGATCTGAAC-3′); D8LΔ-Forward (5′-CGGGGTACCCCGCAACAACTATCTCC-3′) and D8LΔ-Reverse (5′-CCGGGAATTCCTCTCTCAAATCGGACAACCATC-3′); and B5RΔ-Forward (5′-CCGGGTACCACTGTACCCACTATGAATAACG-3′) and B5RΔ-Reverse (5′-CCGGTCGACTGCTTCTAACGATTCTATTTC-3′). The predicted hydrophobic regions of the D8L and B5R proteins were not included in these constructs. The underlined sequences in the primers indicate EcoRI (A27L-, D8LΔ-, and BSRΔ-Forward), KpnI (A27L- and D8LΔ-Reverse), or SalI (BSRΔ-Reverse) enzyme restriction sites. The PCR products were cloned into the pCR2.1 cloning vector, digested with the appropriate restriction enzymes, and ligated into the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible expression vector pET41b, which results in the fusion of glutathione *S*-transferase (GST) with the N terminus of the recombinant protein to facilitate protein purification. The plasmids were subsequently introduced into the *E. coli* protein expression strain Rosetta (A27L) or BL21(DE3) (D8LΔ and B5RΔ). BL21(DE3) transformed with plasmid pET41b was used for the production of GST alone, which was used as a negative control for these studies. Bacterial cultures were induced with IPTG, and the A27L-, B5RΔ-, and D8LΔ-GST fusion proteins or GST alone was purified using glutathione Sepharose 4B (Amersham Biosciences, Piscataway, NJ) according to the manufacturer's protocol. The molecular weight and purity of each recombinant protein were verified by Western blotting and Coomassie staining after resolution by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. An affinity-purified rabbit anti-vaccinia virus IgG antibody (Accurate Chemical and Scientific Corporation, Westbury, NY) was used for Western blotting. The bicinchoninic acid protein assay (Pierce Biotechnology, Rockford, IL) was used to quantitate the concentrations of the proteins. To evaluate the antibody response by enzyme-linked immunosorbent assays (ELISAs), it was necessary to produce purified proteins not fused with GST, in order to avoid the detection of GST-specific humoral responses. For this purpose, digested fragments from the pCR2.1 cloning vectors constructed as described above were cloned into *PLEX* (plasmid-based expression) vectors and transformed into *Streptococcus gordonii*, and the secreted proteins were purified from bacterial culture supernatants, as described previously (51).

Animal immunizations. All animal studies were conducted in accordance with Public Health Service policy and were approved by the Institutional Animal Care and Use Committee of Oregon State University. Six-week-old female BALB/c mice (Charles River, Boston, MA) were immunized three times subcutaneously at weeks 0, 3, and 5 with 10 μg each of A27L-GST, D8LΔ-GST, and B5RΔ-GST or A27L-GST and B5RΔ-GST in 100 μl phosphate-buffered saline (PBS) mixed with 100 μl of monophosphoryl lipid A (MPL)–trehalose dicorynomycolate (TDM) adjuvant (Sigma, St. Louis, MO). After we had performed two immunization experiments, the MPL-TDM adjuvant was no longer commercially available. Hence, a third, confirmatory experiment was performed using TiterMax Gold adjuvant (Sigma). For the identification of linear B-cell epitopes in single-protein-immunized mice (see Fig. 1), mice were immunized twice intraperitoneally with 50 μg of protein in MPL-TDM adjuvant at weeks 0 and 2. For live-

vaccinia virus immunizations, mice were scarified at the base of the tail with 8×10^6 PFU of VV-COP in a 10-μl volume of PBS, as described elsewhere (21).

Vaccinia virus challenge. Five weeks or 2 weeks after the last protein or VV-COP immunization, respectively, mice were lightly anesthetized using 3% isoflurane and challenged intranasally with 20 50% lethal doses (LD_{50}) (~2.4×10^6 PFU) of VV-WR in 20 μl of PBS by applying equal amounts to each of the two nares. The weight and ear temperature (Braun ExacTemp ear thermometer; Welch Allyn, Skaneateles Falls, NY) of each mouse were taken immediately before challenge and on alternate days starting 4 days postchallenge. Mice were also monitored daily for survival and signs of illness (disease index [DI]) and were scored as 0 (normal), 1 (slightly ruffled), 2 (clearly ruffled), 3 (hunched posture and/or conjunctivitis in addition to clear ruffling), 4 (score of 3 combined with difficulty breathing/moving/socializing), and 5 (death), as described elsewhere (35). Those with a DI of 4 and/or a >30% loss of body weight were euthanized.

Sample collection. Five weeks or 2 weeks after the last protein or VV-COP immunization, respectively, five mice from each group were euthanized with CO_2, blood samples were collected by cardiac puncture, and spleens were removed. The blood was incubated at 37°C for 1 h, kept overnight at 4°C to allow clots to contract, and centrifuged at a relative centrifugal force of $9,300 \times g$ for 10 min. The serum was collected and stored at −80°C until use. Single-cell suspensions were prepared from pooled spleens by mashing the spleens gently through 70-μm-pore-size cell strainers (BD Biosciences, San Jose, CA). After osmotic lysis of red blood cells by ACK lysis buffer (0.15 M NH_4Cl, 10 mM $KHCO_3$, 0.1 mM disodium EDTA), the cells were washed in PBS, counted, and resuspended in the appropriate medium for further processing.

IMV neutralization and EEV comet tail inhibition assays. For the IMV neutralization assay, purified VV-WR (50 PFU) was incubated overnight at 37°C with 12 twofold dilutions (starting at 1:25) of heat-inactivated (56°C, 30 min) serum in Dulbecco's minimal essential medium (DMEM) containing 2.5% fetal bovine serum (FBS) and 10 μg/ml gentamicin (DMEM-2.5; all reagents from Invitrogen). Monolayers of BSC-40 (African green monkey kidney) cells in DMEM-2.5 were then infected with the overnight-incubated serum–VV-WR mixture for 4 h at 37°C. After infection, a 1% methylcellulose overlay was added to the wells, and monolayers were incubated at 37°C under 5% CO_2 for 48 h. Finally, the monolayers were stained with 0.1% crystal violet in 30% ethanol for 5 min to enumerate plaques. The percentage of inhibition for each serum dilution was calculated as follows: (average number of plaques in naïve mouse serum − average number of plaques for the immune serum dilution)/(average number of plaques in naïve mouse serum) × 100. Using XLfit curve-fitting software for Excel (ID Business Solutions Inc., Alameda, CA), the plaque reduction neutralization titers, or 50% inhibitory concentrations (IC_{50}), were then determined as the reciprocal of the highest dilution resulting in 50% inhibition of plaque formation. For comet inhibition assays, monolayers of BSC-40 cells in six-well plates were first infected with ~100 PFU of VV-IHD-J in MEM-5.0 for 1 h at 37°C. Subsequently, the viral inoculum was removed by aspiration and replaced with a 1-ml liquid overlay of MEM-5.0 containing 40 μl (final dilution, 1:25) of serum. The plates were incubated at 37°C under 5% CO_2 for an additional 35 h. The cells were then fixed with 5% glutaraldehyde in PBS and stained with 0.1% crystal violet in 5% methanol to visualize plaques and comet tails.

ELISA. For protein-specific serum ELISAs, 96-well MaxiSorp surface plates (Nalge Nunc International, Rochester, NY) were coated with 500 ng/well of A27L, D8LΔ, or B5RΔ protein purified from *Streptococcus gordonii*. For the vaccinia virus-specific ELISA, plates were coated with 1×10^6 PFU/well of purified VV-IHD-J. The virus was inactivated by UV light cross-linking using five consecutive 1-min cycles in a 125-mJ UV chamber (GS Gene Linker; Bio-Rad Laboratories, Hercules, CA). The plates were then washed in PBS–0.05% Tween 20 and blocked with PBS–10% FBS for 1 h at room temperature (RT). Threefold serial dilutions of pooled sera from five mice/group in PBS–10% FBS were added to the blocked plates and incubated at RT for 2 h. After a wash, horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (diluted 1:10,000; Bio-Rad, Hercules, CA), IgG1, or IgG2a (each diluted 1:5,000; Bethyl Laboratories, Montgomery, TX) antibody in PBS–10% FBS was added to the wells and incubated at RT for 1 h. After a wash, the ELISA was developed by adding a 1:1 mixture of tetramethyl benzidine and hydrogen peroxide (Pierce Biotechnology, Rockford, IL). Color development was stopped by using 2N sulfuric acid, and the absorbance was read at 450 nm in a Spectrafluor Plus plate reader (Tecan, San Jose, CA), with a reference filter set at 620 nm.

Peptide screening for linear B-cell epitopes. Biotinylated A27L-, B5R-, and D8L-derived peptides spanning the lengths of the immunizing proteins were synthesized by Sigma-Genosys (St. Louis, MO) (see Table S1 in the supplemental material). The peptides were 20 aa in length and contained 10-aa overlaps between contiguous peptides. A number of B5R and D8L peptides could not be synthesized. NeutrAvidin-coated and bovine serum albumin-blocked plates (Pierce Biotechnology) were washed with wash buffer (1× PBS, 0.1% bovine serum albumin, 0.05% Tween 20) before addition of 500 ng/well of peptide diluted in wash buffer. After overnight incubation at 4°C, plates were washed, and diluted sera were added to wells. After 3 h of incubation at RT, the plates were washed, and an HRP-conjugated goat anti-mouse IgG antibody (1:10,000 dilution) was added for 1 h. Plates were developed as described in the preceding section.

Peptide screening for T-cell epitopes. Peptides (see Table S1 in the supplemental material) were synthesized without biotinylation. A few peptides from the A27L, B5R, and D8L proteins were not included in the screening, either because they could not be synthesized or because they were not part of the proteins used for immunization. $OVA_{323–339}$ peptide (24) was used as a negative control. Red blood cell-depleted splenocytes were first resuspended in complete medium (CM; RPMI 1640 containing 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin sulfate, 50 μM 2-mercaptoethanol, and 10 mM HEPES), and 1×10^6 cells/well were added to MultiScreen 96-well assay plates (Millipore, Billerica, MA), which had been coated overnight at 4°C with purified anti-IFN-γ antibodies and washed and blocked with CM at RT for 2 h. The splenocytes were then stimulated with 10 μg/ml of $OVA_{323–339}$ peptide or protein-specific peptides for 36 to 40 h at 37°C under 5% CO_2. At the end of the culture period, the supernatant was aspirated, and the plates were washed with deionized water and wash buffer (PBS–0.05% Tween 20). The plates were incubated with biotinylated anti-IFN-γ followed by HRP-conjugated streptavidin, and then IFN-γ spots were developed using 3-amino-9-ethylcarbazole substrate solution as recommended by the manufacturer (BD Biosciences). The plates were sent to Cellular Technology Limited (Cleveland, OH) for blinded enumeration of spots. When the data were analyzed, the number of spots in no-peptide wells was considered background IFN-γ secretion and was subtracted from that in peptide-stimulated wells. For the confirmation of the specificity of the peptides by stimulation of $CD4^+$ or $CD8^+$ T cells, these T cells were selected using CD4 or CD8 MicroBeads according to the manufacturer's instructions (Miltenyi Biotec, Auburn, CA). The purity of positively selected T cells, as assessed by flow cytometry, was greater than 96% (data not shown). $CD4^+$ or $CD8^+$ T cells were resuspended in CM, and 2.0×10^5 cells/well were added to anti-IFN-γ antibody-coated enzyme-linked immunospot (ELISPOT) plates. To serve as antigen-presenting cells, 6.0×10^5 naïve splenocytes were added per well after treatment with 50 μg/ml mitomycin C for 20 min at 37°C and triple washing. Stimulation and plate development were performed as described for unfractionated splenocytes.

Intracellular cytokine staining (ICCS). One million unfractionated splenocytes/well were stimulated with 10 μg/ml of peptide in 96-well round-bottom plates (Corning, Corning, NY) at 37°C under 5% CO_2 for 13 h in the presence of 3 μg/ml of brefeldin A (eBioscience, San Diego, CA). The cells were then blocked with anti-mouse CD16/32 antibodies, surface stained with phycoerythrin-Cy5-conjugated anti-CD4 or fluorescein isothiocyanate-conjugated anti-CD8 antibodies (all from BD Biosciences), fixed with 4% paraformaldehyde (eBioscience), permeabilized, stained with a phycoerythrin-conjugated anti-IFN-γ antibody (Pharmingen), and analyzed by flow cytometry. A Cytomics FC 500 flow cytometry system (Beckman Coulter, Fullerton, CA) at Oregon State University was used for data acquisition, and WinMDI software (The Scripps Research Institute, La Jolla, CA) was used for data analysis.

Passive transfer studies. Mice were vaccinated with three doses of GST or with combinations of A27L-GST, D8L-GST, and B5R-GST proteins in MPL-TDM adjuvant, as described above. Immune sera and splenocyte single-cell suspensions were prepared 5 weeks after protein immunization or 2 weeks after live VV-COP immunization. To assess whether the adoptive transfer of splenocytes confers protection from virus challenge, 5 to 10 naïve mice were intravenously administered 2×10^7 splenocytes in 500 μl of PBS 24 h prior to intranasal challenge with 20 LD_{50} of VV-WR. For the assessment of the role of antibodies in protection, mice were intravenously injected with 500 μl of immune serum 3 h prior to and 24 h after challenge with VV-WR. Mice were monitored for survival, maintenance of weight and temperature, and signs of illness as described above.

Statistical analyses. For comparison of two groups, an unpaired Student *t* test was used and *P* values of <0.05 were considered statistically significant. One-way analysis of variance with a Holm-Sidak correction was used for comparing more than two groups. SigmaStat 3.1 software (San Jose, CA) was used for performing all statistical analyses.

RESULTS

Immunization of mice with *E. coli*-expressed A27L, D8LΔ, and B5RΔ proteins induces high-titer antibody responses. Vaccines that are composed of the outer membrane proteins

TABLE 1. Antibody responses of mice vaccinated with *E. coli*-expressed vaccinia virus antigens

Target antigen	Antibody	End point titer[a] of antibody or IC_{50}[b] with the following vaccine (adjuvant):					
		GST (MPL + TDM)	A27L + B5R + D8L (MPL + TDM)	GST (TiterMax)	A27L + B5R (TiterMax)	A27L + B5R + D8L (TiterMax)	VV-COP
A27L[a]	IgG	556	405,000	185	1,215,000	1,215,000	900
	IgG1	185	405,000	<185	1,215,000	1,215,000	<100
	IgG2a	<185	135,000	<185	135,000	135,000	300
B5R[a]	IgG	1,667	1,215,000	185	405,000	405,000	2,700
	IgG1	185	405,000	185	1,215,000	1,215,000	100
	IgG2a	185	405,000	<185	45,000	135,000	900
D8L[a]	IgG	556	1,215,000	185	135,000	405,000	72,900
	IgG1	185	1,215,000	<185	135,000	405,000	2,700
	IgG2a	<185	405,000	<185	5,000	135,000	72,900
VV-IHD-J[a,c]	IgG	5,000	1,215,000	1,667	3,645,000	3,645,000	40,500
	IgG1	<185	405,000	<185	1,215,000	1,215,000	<185
	IgG2a	185	405,000	185	405,000	405,000	40,500
VV-WR[b]		<25	7,632	<25	1,643	3,763	50

[a] Reciprocal of the serum dilution 3 standard deviations above the average optical density of assay diluent wells containing no mouse serum. The capture antigens used for individual protein ELISA were purified from *Streptococcus gordonii* and do not contain GST. The IgG end point titer represents the combination of all isotypes.
[b] Reciprocal of the serum dilution that inhibits VV-WR plaque formation by 50%.
[c] ELISA plates were coated with purified VV-IHD-J and inactivated by UV light cross-linking.

of both IMV and EEV have been shown to provide superior protection against lethal vaccinia virus challenge compared to vaccines containing individual IMV or EEV proteins (14, 15, 19, 21, 41, 55). Soluble recombinant vaccinia virus proteins produced in either insect cells (14, 15) or *E. coli* (8, 27, 28) have been demonstrated to be immunogenic and protective. In this study, the IMV-associated proteins A27L and D8L and the EEV-associated protein B5R were produced in *E. coli* and confirmed by Coomassie blue staining and Western blotting using a commercially available purified rabbit anti-vaccinia virus IgG antibody (data not shown) or sera from VV-COP-immunized mice (data not shown). Under nonreducing conditions, D8LΔ-GST appears as a monomer, A27L-GST as trimers, and B5RΔ-GST as oligomers (data not shown). It was also of interest to determine whether the *E. coli*-expressed vaccinia virus proteins can be recognized by human immune serum, since antibody responses to A27L, D8L, and B5R are induced in humans after immunization with live smallpox vaccine (8, 36). Western blot analysis showed that the A27L, D8L, and B5R proteins expressed in *E. coli* did not react with human preimmune serum but were reactive with human serum collected 4 weeks after vaccination with Dryvax (data not shown).

The immunogenicity of the recombinant proteins was examined by subcutaneously immunizing BALB/c mice three times with 10 μg each of either GST-fused A27L and B5R proteins, GST-fused A27L, D8L, and B5R proteins, or purified GST (as a negative control) in MPL-TDM or TiterMax Gold adjuvant. Mice scarified once on their tails with live VV-COP served as a positive-control group. Five weeks after the last protein immunization or 2 weeks after vaccinia virus scarification, the mice were bled, and the serum was used to assess the induction of protein- or vaccinia virus-specific antibodies. High levels of A27L-, D8L-, or B5R-specific IgG antibody responses were detected in the sera of mice immunized with the two-protein (A27L and B5R) or three-protein (A27L, D8L, and B5R) combination (Table 1). Sera from GST-immunized mice did not contain antibodies that reacted with any of the three proteins, suggesting that the observed antibody responses to the ELISA coating antigens are specific to the immunizing proteins and are not due to anti-*E. coli* or cross-reactive anti-GST antibodies. Most importantly, the sera of protein-immunized mice, but not those of GST-immunized mice, contained very high titers of antibody that cross-reacted with VV-IHD-J. These data indicate that immunization with a subunit vaccine consisting of vaccinia virus outer membrane proteins produced in bacteria can generate a robust humoral response that targets the virus. In addition, the end point titer of the antibody response against the virus was higher than the titers of antibodies to individual component proteins of the vaccine administered in TiterMax adjuvant. Immunization with the A27L and B5R proteins in TiterMax adjuvant generated levels of anti-vaccinia virus antibody response similar to those for immunization with A27L, B5R, and D8L, implying that A27L and B5R alone might be sufficient as vaccine components. Interestingly, reactivity to D8L was higher than background (GST) in A27L- and B5R-immunized mice, suggesting that vaccination with the latter two proteins may induce some antibodies that cross-react with D8L. Vaccination of mice with live vaccinia virus induced antibody responses to all three proteins and VV-IHD-J, although the levels were significantly lower than those detected for recombinant-protein-immunized mice.

Both the MPL-TDM and TiterMax adjuvants were effective at inducing high levels of protein- and virus-specific antibody responses detected by ELISA. However, in mice immunized with proteins in TiterMax, there were higher levels of IgG1 than IgG2a isotype antibodies to each protein and to VV-IHD-J, suggesting that this adjuvant may stimulate a T helper 2 (Th2)-polarized humoral response. In contrast, immunization with proteins in MPL-TDM induced similar levels of IgG1 and IgG2a antibodies to B5R, D8L, and VV-IHD-J, suggesting

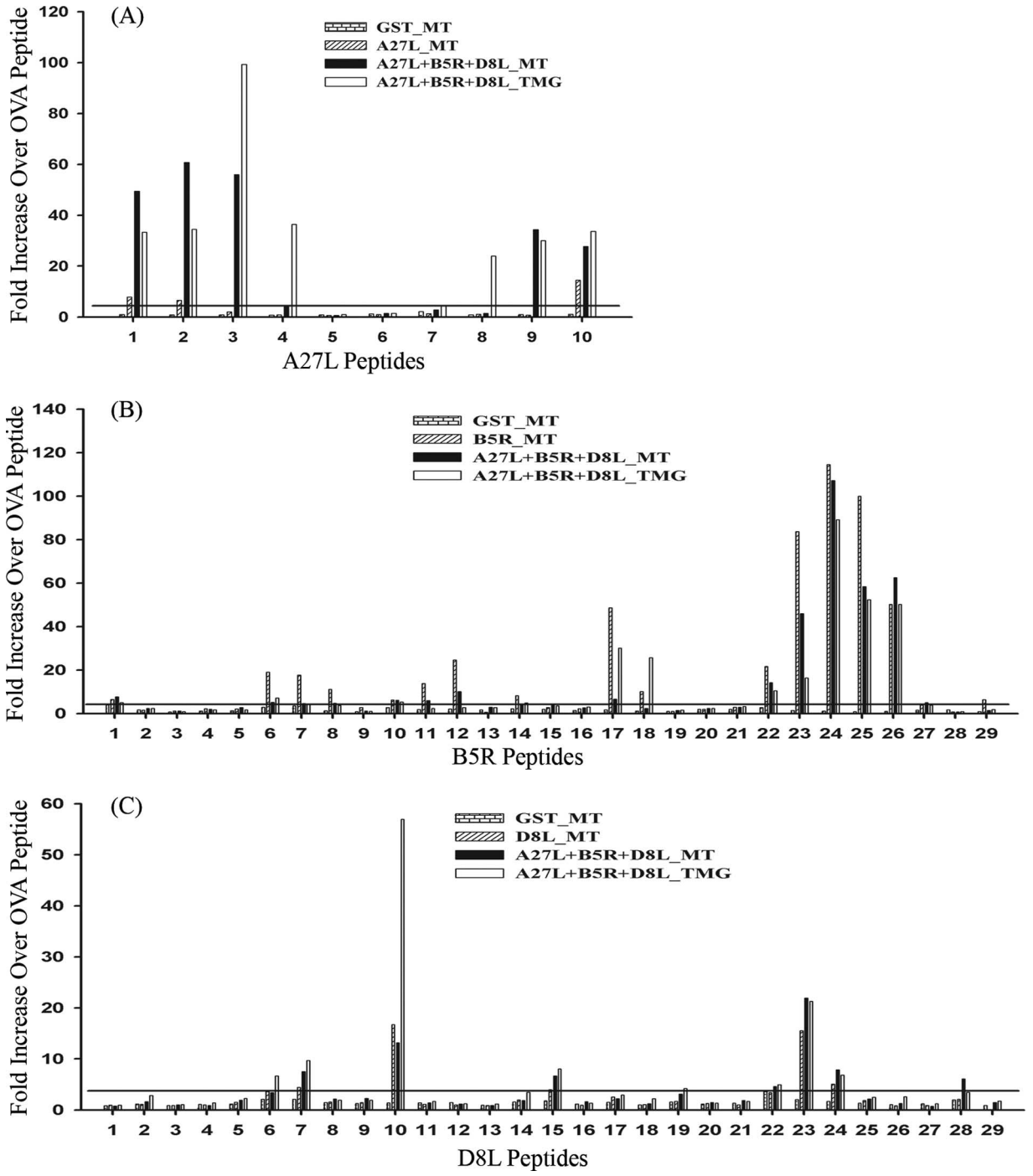

FIG. 1. Reactivities of recombinant-protein immune sera with potential linear B-cell epitopes within the vaccinia virus A27L (A), B5R (B), and D8L (C) proteins. BALB/c mice received a total of three (weeks 0, 3, and 5) subcutaneous vaccinations of 10 μg of GST or a combination of A27L-GST, D8LΔ-GST, and B5RΔ-GST proteins in MPL-TDM (MT) or TiterMax Gold adjuvant. Serum samples were obtained from these mice 5 weeks after the last immunization or from mice immunized with individual proteins twice and sacrificed 2 weeks after the second vaccination. Twenty-amino-acid biotinylated peptides were added to NeutrAvidin-coated plates and used to screen the serum samples by ELISA. Data are presented as the increase in the absorbance (at 450 nm) of each peptide over that of an irrelevant peptide, $OVA_{323–339}$. Serum reactivity levels fourfold (indicated by horizontal line) or more higher than that with the OVA peptide were considered positive.

that this adjuvant may not preferentially skew the humoral response to these antigens to either Th1 or Th2. Interestingly, as with TiterMax, the administration of A27L in MPL-TDM appears to induce more IgG1 than IgG2a isotype antibodies, suggesting that the effects of the adjuvants might be antigen dependent. Live-vaccinia virus immunization resulted in a Th1-polarized humoral response, inducing mainly IgG2a isotype antibody responses, as previously reported (37).

Recombinant A27L, D8LΔ, and B5RΔ protein immunization induces antibodies targeting multiple sites of the individual proteins. In order to assess regions of the immunizing proteins that are recognized by antibodies generated by vaccination, sera from immunized mice were screened by ELISA for reactivity against peptides derived from A27L, D8L, and B5R. Recombinant-protein serum reactivity was considered positive if the increase over the reactivity to $OVA_{323–339}$ was greater than fourfold, i.e., 3 standard deviations above the average for all the peptides incubated individually with GST serum. By this criterion, sera from mice immunized with the triple-protein combination in MPL-TDM adjuvant reacted with A27L peptides 1 to 4 (aa 1 to 50) and 9 and 10 (aa 81 to 110) (Fig. 1A); B5R peptides 6 to 8 (aa 51 to 90), 10 to 12 (aa 101 to 140), 14 (aa 141 to 160), 17 (aa 171 to 190), and 22 to 27 (aa 221 to 290) (Fig. 1B); and D8L peptides 7 (aa 61 to 80), 10 (aa 91 to 110), 15 (aa 151 to 170), and 22 to 24 (aa 231 to 270) (Fig. 1C). Similar peptide reactivities were observed in the sera of mice vaccinated with the triple proteins in TiterMax, with a few exceptions: There was additional reactivity to A27L peptides 7 and 8 (aa 61 to 90), B5R peptide 18 (aa 181 to 200), and D8L peptides 6 (aa 51 to 70) and 19 (aa 201 to 220), whereas there was no reactivity to B5R peptides 8 (aa 71 to 90), 11 to 12 (aa 111 to 140), and 27 (aa 271 to 290). In addition, antibody reactivities to several of the peptides of a specific protein were detectable in the sera of the triple-protein-immunized mice as well as in sera from mice immunized intraperitoneally with the applicable protein alone (Fig. 1A to C). This indicates that each protein is immunogenic on its own, in the absence of the other proteins. Perhaps due to the low end point titers of the antibody response (Table 1), the screening of the peptides using sera from VV-COP-immunized mice did not detect peptide reactivity above background levels in preimmune serum except for the low reactivity of B5R peptides 8 (aa 71 to 90) and 24 (aa 241 to 260) (data not shown). These data imply that recombinant-protein immunization can induce the generation of significant levels of heterogeneous antibodies that recognize linear epitopes from multiple regions of the protein.

Antibodies generated by vaccination with recombinant A27L, D8LΔ, and B5RΔ proteins possess vaccinia virus-neutralizing activity. The vaccinia virus A27L and D8L proteins are known to elicit IMV-neutralizing antibodies, and these antibodies have been correlated with protection against live-vaccinia virus challenge in mice (8, 23, 27). Because high levels of A27L- and D8L-specific antibodies were detected in the sera of protein-immunized mice (Table 1), IMV neutralization assays were performed to determine whether sera from these mice could inhibit the infection of tissue culture cells by VV-WR. No virus neutralization activity was detected in the sera of mice immunized with GST in either MPL-TDM or TiterMax adjuvant. The highest level of IMV-neutralizing activity was found in the sera of mice immunized with all three proteins in MPL-TDM adjuvant; it was twofold higher than that with the proteins delivered in TiterMax adjuvant (Table 1). The virus-neutralizing activity of sera from mice immunized with A27L, D8L, and B5R was twofold higher than that for mice immunized with A27L and B5R alone. These data suggest that antibodies against D8L may synergize with antibodies against A27L for maximal neutralization of viral infectivity. The IMV-neutralizing activities of mouse sera obtained 2 weeks after vaccination with vaccinia virus were 32- to 152-fold lower than those of the sera of recombinant-protein-immunized mice. This may be because higher levels of neutralizing antibodies induced by dermal scarification with live vaccinia virus become detectable at 3 weeks postimmunization (14).

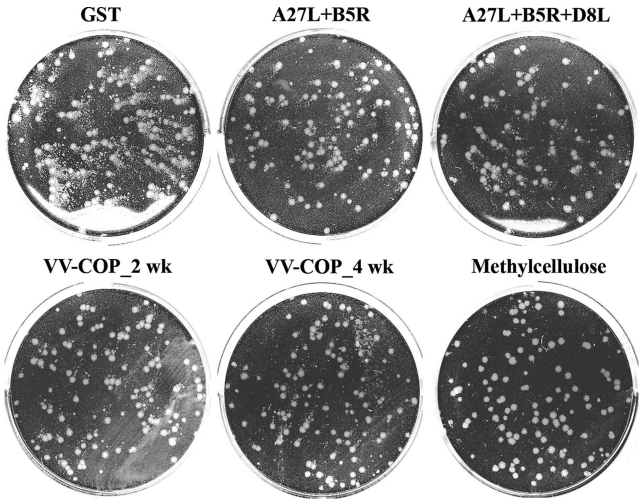

FIG. 2. Sera from mice immunized with *E. coli*-expressed vaccinia virus recombinant proteins inhibit EEV spread to distal cells and comet tail formation in vitro. Serum samples were obtained from mice vaccinated with either GST, the two-protein combination of A27L and B5R, or the three-protein combination of A27L, D8L, and B5R in TiterMax Gold adjuvant as described in the legend to Fig. 1. For comparison, mice were vaccinated once with 8×10^6 PFU of VV-COP by dermal scarification, and serum samples were collected 2 or 4 weeks later. Monolayers of BSC-40 cells were infected with ~100 PFU of VV-IHD-J, aspirated to remove the viral inoculum, and overlaid with liquid medium containing a 1:25 final dilution of the indicated serum. After incubation for an additional 35 h, the cells were fixed and stained with 0.1% crystal violet to visualize plaques and comet tails. Wells containing cells infected with virus but overlaid with methylcellulose were used as controls for complete inhibition of EEV dissemination.

The release of large amounts of EEV from cells infected with VV-IHD-J leads to the formation of comet-shaped plaques in monolayers of tissue culture cells (26, 46). Comet formation can be inhibited by the presence of EEV-targeting antibodies that prevent virus release from infected cells, including those directed against B5R (49). The sera of mice that had been scarified or immunized with proteins were tested to determine whether antibodies capable of inhibiting comet formation were induced after vaccination. The liquid-overlay comet inhibition assay for which results are shown in Fig. 2 demonstrates that sera from GST-immunized mice were not capable of inhibiting the in vitro dissemination of EEV from infected cells and thus resulted in the formation of several small, satellite plaques. However, satellite plaque formation was significantly inhibited by sera from mice vaccinated with VV-COP or combinations of the two or three proteins in either MPL-TDM (data not shown) or TiterMax adjuvant (Fig. 2). These data imply that recombinant-protein immunization stimulates the production not only of IMV-neutralizing antibodies but also of antibodies that are capable of preventing long-range virus dissemination.

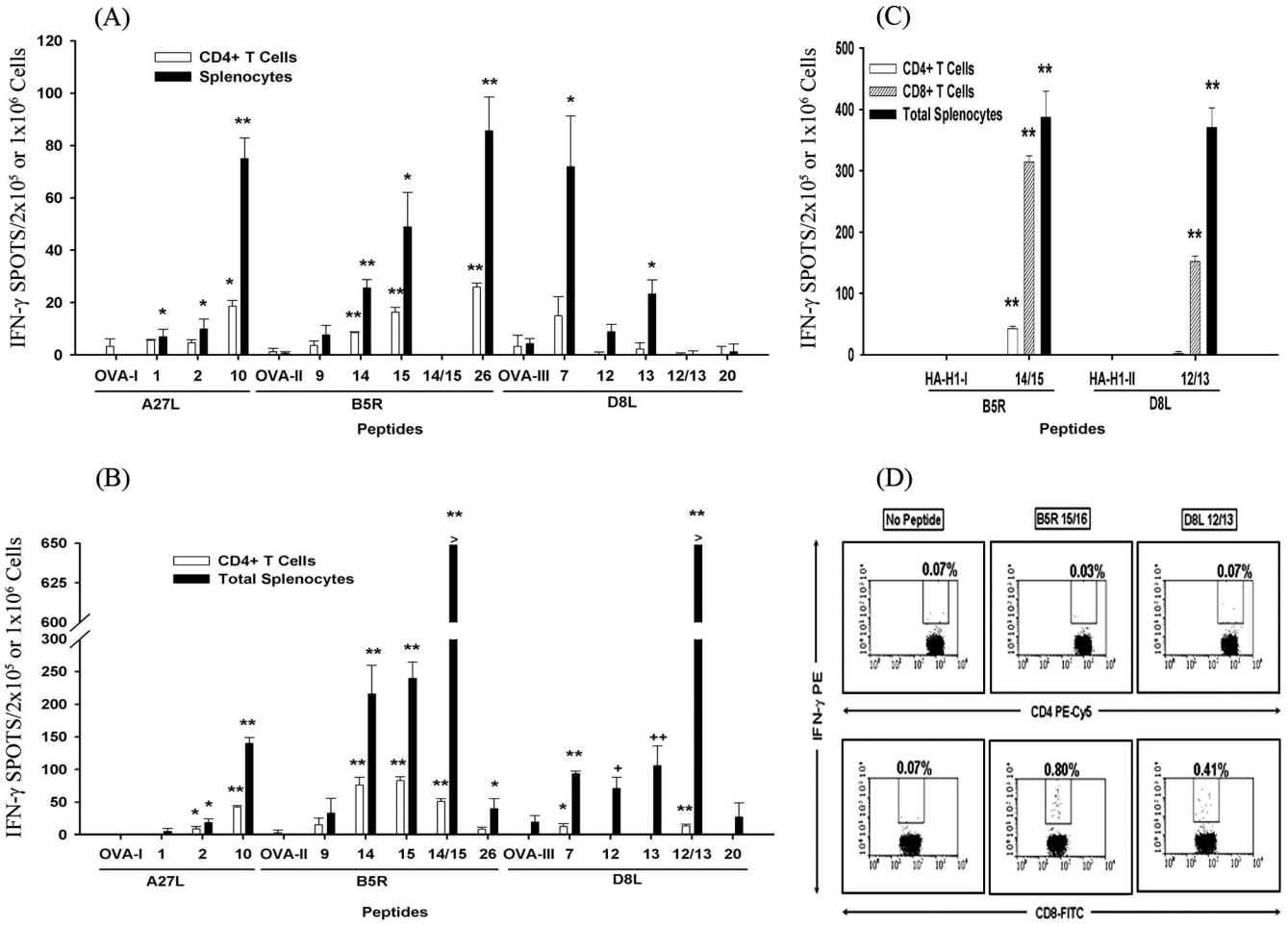

FIG. 3. T-cell responses induced by vaccination with recombinant proteins (A) or VV-COP (B to D). Mice were immunized with A27L, D8LΔ, and B5RΔ proteins in TiterMax adjuvant or with VV-COP, as described in the legends to Fig. 1 and 2, respectively. Five weeks after the last dose of protein immunization (A) or 2 (B and C) or 4 (D) weeks after VV-COP dermal scarification, spleens were obtained, and unfractionated splenocytes or positively selected $CD4^+$ or $CD8^+$ T cells were stimulated with 10 μg/ml of the indicated peptide. Mitomycin C-treated naïve splenocytes were used as antigen-presenting cells for the stimulation of $CD4^+$ or $CD8^+$ T cells. Data in panels A to C represent mean numbers of IFN-γ-positive spots in triplicate wells. Error bars, standard errors of the means. *, $P < 0.05$; **, $P < 0.01$; +, $P = 0.059$; ++, $P = 0.055$. For ICCS (D), unfractionated splenocytes from mice vaccinated with VV-COP (4 weeks earlier) were stimulated for 13 h with no peptide or with overlap peptides in the presence of brefeldin A. The cells were then surface stained with anti-CD4 or anti-CD8 antibodies and intracellularly stained with anti-IFN-γ antibody. The percentage of $CD4^+$ or $CD8^+$ T cells responding to the stimulation by producing IFN-γ (given above each plot) was quantitated by flow cytometry.

Cellular responses stimulated by recombinant A27L, D8LΔ, and B5RΔ protein immunization. Both humoral and cellular immune responses can contribute to immunity against vaccinia virus infections (56). Given the important role $CD4^+$ T cells can play in the generation and maintenance of humoral and $CD8^+$ T-cell responses, the induction of peptide-specific cellular responses in protein-immunized mice was analyzed by using peptides derived from the A27L, B5R, and D8L proteins. We first identified potential T-cell epitopes specific for each protein by an ELISPOT screen of splenocytes obtained from mice that had received two intraperitoneal immunizations of each protein alone in MPL-TDM adjuvant or mice that had been scarified with VV-COP and intranasally challenged with VV-WR (data not shown). Splenocyte stimulation at least twofold higher than that with $OVA_{323–339}$ was detected for A27L peptides 1 (aa 1 to 20), 2 (aa 11 to 30), and 10 (aa 91 to 110), B5R peptides 9 (aa 81 to 100), 14 (aa 141 to 160), 15 (aa 151 to 170), and 26 (aa 261 to 280), and D8L peptides 7 (aa 61 to 80), 12 (aa 121 to 140), 13 (aa 131 to 150), and 20 (aa 211 to 230). These peptides were used to compare the cellular response elicited by vaccination with combinations of A27L, D8L, and B5R proteins with that for VV-COP. In triple-protein-immunized mice, statistically significant ($P < 0.05$) (Fig. 3A) numbers of peptide-specific splenocytes were detected for A27L peptides 1, 2, and 10, B5R peptides 14, 15, and 26, and D8L peptides 7 and 13. The responses to A27L peptides 2 and 10, B5R peptides 14, 15, and 26, and D8L peptides 7, 12, and 13 in VV-COP-immunized mice were also statistically significant ($P < 0.05$) or nearly significant ($P < 0.06$) (Fig. 3B). We anticipated that the peptide-specific splenocytes were $CD4^+$ T cells rather than $CD8^+$ T cells. This is because the induction of $CD8^+$ T cells via the exogenous pathway would

require cross-presentation of endocytosed recombinant protein, which occurs only under certain circumstances, such as the presence of stimuli that trigger dendritic cell maturation (1). In addition, the peptides used for stimulation were 20 aa long and would require processing to 8 to 10 aa in vitro in order to be recognized by $CD8^+$ T cells. To assess whether the IFN-γ detected after peptide stimulations of splenocytes was produced by $CD4^+$ T cells, the T cells were positively selected and stimulated with peptides in the presence of naïve splenocytes. Statistically significant $CD4^+$ T-cell responses were detected in either protein- or VV-COP-vaccinated mice for A27L peptides 2 and 10, B5R peptides 14, 15, and 26, and D8L peptide 7 (Fig. 3A and B), suggesting that these peptides contain $CD4^+$ T-cell epitopes. Although $CD4^+$ T-cell responses were not evident for D8L peptides 12 and 13 in the experiment for which results are shown in Fig. 3B, statistically significant ($P < 0.027$; peptide 13) or near-significant ($P < 0.066$; peptide 12) $CD4^+$ T-cell responses were detected in a repeat screening (data not shown). Furthermore, no IFN-γ-producing $CD8^+$ T cells were detected when positively selected CD8 T cells were stimulated with any of the 20-mer A27L, B5R, or D8L peptides characterized above (data not shown).

$CD8^+$ T-cell epitopes present within aa 141 to 170 of B5R protein and aa 121 to 150 of D8L protein. As shown in Fig. 3A and B, T-cell responses were detected in B5R peptides 14 and 15 and D8L peptides 12 and 13, which are contiguous and have 10-aa overlaps in their sequences. Hence, we synthesized the 10-aa-overlap peptides to test whether this minimal epitope can stimulate $CD4^+$ T cells. Neither unfractionated splenocytes nor $CD4^+$ T cells from triple-protein-immunized mice responded to stimulation with B5R 14/15 or D8L 12/13 overlap peptides (Fig. 3A). However, statistically significant $CD4^+$ T-cell responses to both overlap peptides were detected in mice immunized with VV-COP, although the number of responder $CD4^+$ T cells was >12-fold lower for B5R 14/15 and >47-fold lower for D8L 12/13 than the number of responders in unfractionated splenocytes (Fig. 3B). Interestingly, when the CD4-depleted splenocytes (flowthrough of CD4 MicroBead selection) from VV-COP-vaccinated mice were stimulated with the overlap peptides, the number of IFN-γ-producing responder cells was as high as that seen for the unfractionated splenocytes (data not shown). This suggested that the majority of the T cells responding to the overlap peptides were most likely $CD8^+$ T cells. To test this, the responses of positively selected CD4 or CD8 T cells were directly compared to those of unfractionated splenocytes (Fig. 3C). A 10-mer peptide derived from the influenza virus hemagglutinin (HA-H1; aa 111 to 120) was used as a negative control, and as expected, no IFN-γ-producing $CD8^+$ T cells were detected upon stimulation with it. In contrast, high numbers of $CD8^+$ T cells were detected upon stimulation with B5R 14/15 or D8L 12/13, confirming our speculation that these overlap peptides are indeed $CD8^+$ T-cell epitopes. The peptide stimulation for the ELISPOT data shown in Fig. 3C was performed using splenocytes obtained 2 weeks after vaccination with VV-COP. We also assessed by ICCS whether the peptide-specific $CD8^+$ T cells are maintained as memory T cells, by examining the T-cell response on day 28 postvaccination, since it has been shown that the $CD8^+$ T-cell response detected at day 30 is maintained at the same level for >300 days (18). As shown in Fig. 3D, the percentage of $CD4^+$ T cells that produced IFN-γ upon peptide stimulation was the same as or below the background level detected in $CD4^+$ T cells that were not stimulated with any peptide. However, the addition of B5R 14/15 or D8L 12/13 peptides stimulated IFN-γ production in 0.8% and 0.41% of $CD8^+$ T cells, respectively, confirming that the overlap peptides contain epitopes recognized by memory $CD8^+$ T cells.

Immunization with recombinant A27L, D8LΔ, and B5RΔ proteins protects mice from lethal vaccinia virus challenge. To test the capability of the immune response induced by vaccination with recombinant proteins to provide protection against viral disease, vaccinated mice were intranasally challenged with 20 LD_{50} of VV-WR 5 weeks after the last immunization. VV-COP-immunized mice were challenged with the same lethal dose of VV-WR 2 weeks after dermal scarification with the virus. Challenged mice were then monitored for survival, maintenance of prechallenge body weight and temperature, and physical signs of illness. All the mice that received either A27L and B5R or A27L, D8L, and B5R proteins in either MPL-TDM (Fig. 4A) or TiterMax (Fig. 4E) adjuvant, or live-vaccinia virus immunization (Fig. 4A and E), survived the lethal virus challenge, whereas all of the mice vaccinated with GST alone succumbed within 5 to 8 days post-viral challenge. Although all of the protein-immunized mice survived virus challenge, they exhibited continuous weight loss, like GST-immunized mice, until day 6 postchallenge, but unlike GST-immunized mice, they regained their starting weight by days 10 to 14 (Fig. 4B and F). However, even on the day of the greatest weight loss (day 6 postchallenge), the recombinant-protein-immunized mice did not lose more than an average of 20% of their starting weight, and they never reached the 30% cutoff designated as the humane end point for euthanasia. In contrast, none of the mice immunized with VV-COP exhibited a weight loss of >4% of their starting weight. Compared to that of VV-COP-immunized mice, the weight loss observed for mice immunized with the proteins in MPL-TDM adjuvant was statistically significant ($P < 0.025$) from days 4 to 14. On the other hand, the weights of mice immunized with recombinant proteins in TiterMax adjuvant were statistically different ($P < 0.015$) from the weights of VV-COP-immunized mice only on day 8, suggesting that immunization in TiterMax adjuvant may provide better protection against weight loss than immunization in MPL-TDM. Further, with MPL-TDM adjuvant immunizations, the weight loss observed for mice immunized with A27L and B5R was statistically different ($P < 0.05$) from the weight loss for the triple-protein-immunized mice on days 6 to 10. However, when TiterMax adjuvant was used, there was no statistical difference in weight loss between mice immunized with A27L and B5R and mice immunized with A27L, D8L, and B5R on any day postchallenge, implying that the two-protein combination can be equally efficacious if the right adjuvant is used to formulate the vaccines.

In previous studies, we have observed that naïve mice challenged intranasally with 20 LD_{50} of VV-WR showed a continuous decrease in ear temperature that paralleled their weight loss, while VV-COP-immunized mice maintained both ear temperature and body weight. In general, an ear temperature below 33°C is a good predictor of severe illness and impending death, especially if it occurs in conjunction with declines in

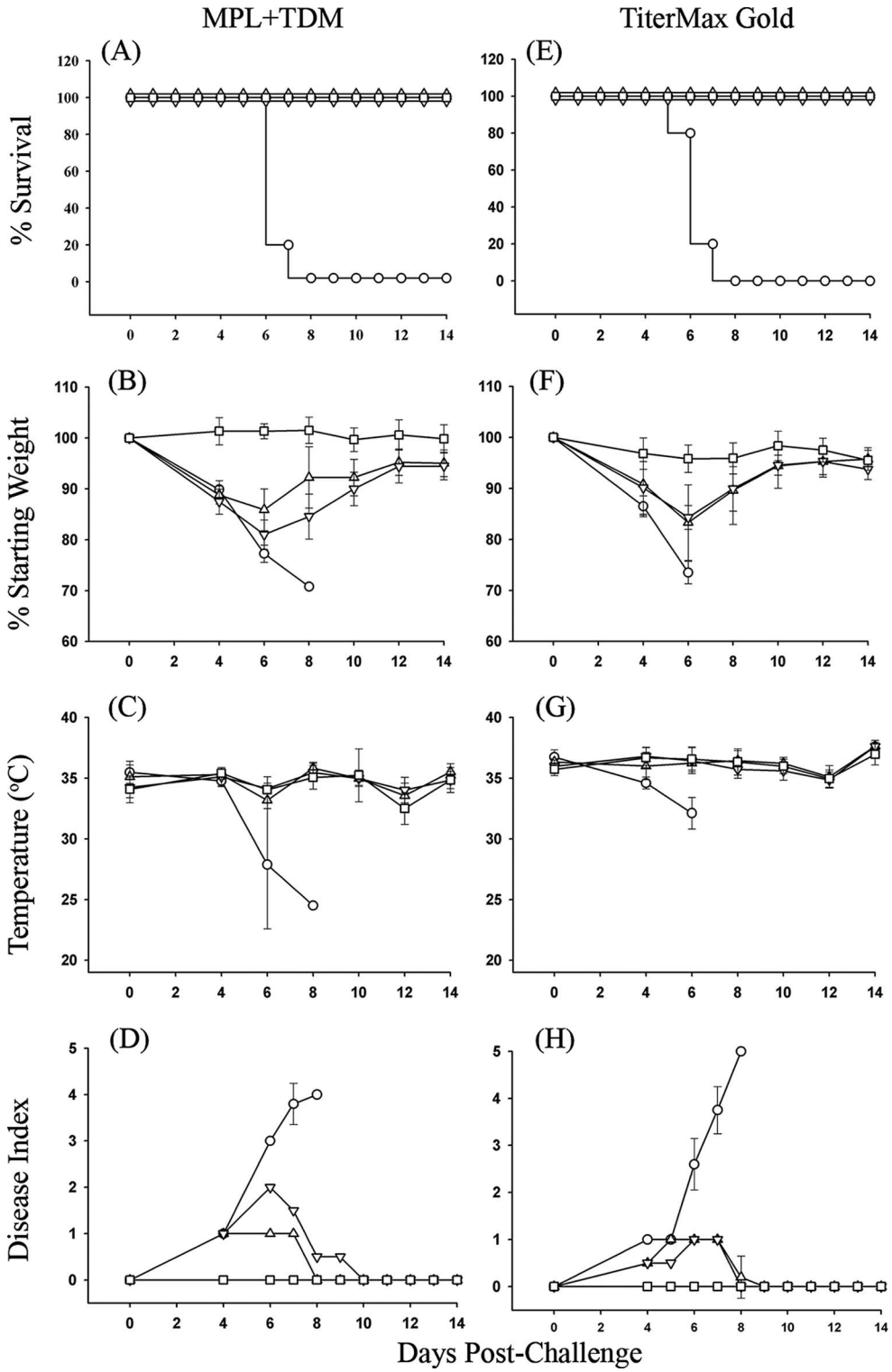

FIG. 4. Immunization of mice with A27L, D8LΔ, and B5RΔ proteins provides full protection from lethal vaccinia virus challenge. Mice (five per group) were vaccinated with recombinant proteins administered in MPL-TDM (A to D) or TiterMax (E to H) adjuvant, as described for Fig. 1. One group of five mice was immunized with a control protein, GST, while another group was vaccinated with VV-COP by dermal scarification. At week 10 for GST- or recombinant-protein-immunized mice or at week 2 for VV-COP-vaccinated mice, the mice were challenged intranasally with 20 LD_{50} of VV-WR (~2.4×10^6 PFU) in 20 μl of PBS. Survival (A and E), weight loss (B and F), ear temperature (C and G), and level of illness (D and H) were recorded for each mouse and were plotted as group averages ± standard deviations. Symbols: ○, GST; △, A27L plus B5R; ▽, A27L, B5R, and D8L; □, VV-COP.

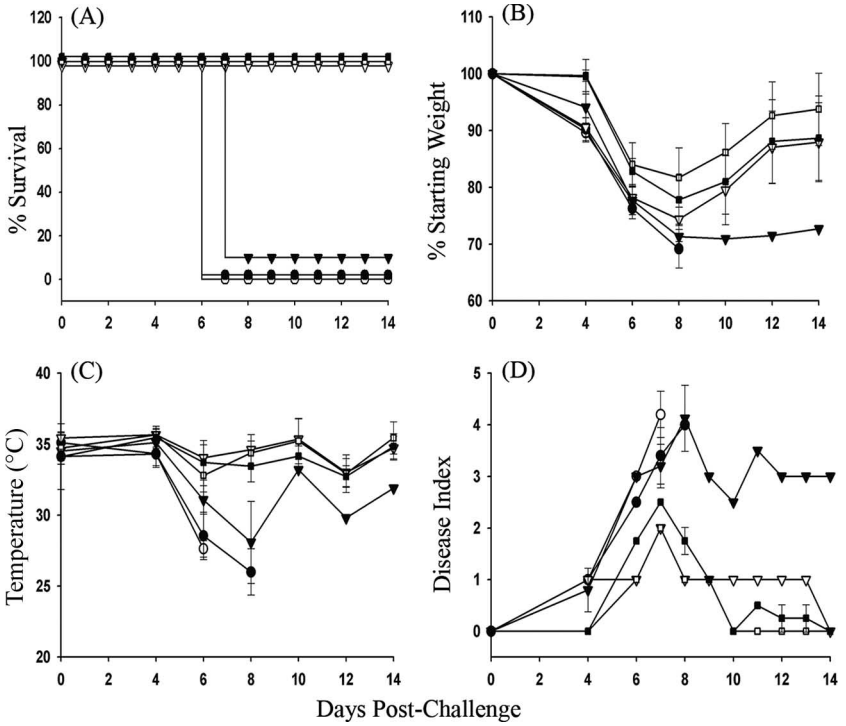

FIG. 5. Passive transfer of protein immune serum, but not of splenocytes, protects mice from lethal vaccinia virus challenge. Sera or splenocytes prepared from mice vaccinated with recombinant protein or VV-COP, as described for Fig. 1 and 2, respectively, were used. A group of 5 to 10 naïve mice were given 2×10^7 splenocytes intravenously 24 h prior to virus challenge. Mice that received serum were administered 500 μl of serum 3 h before and 24 h after virus challenge. Virus challenge was performed as described for Fig. 4. Survival (A), weight loss (B), ear temperature (C), and level of illness (D) were recorded for each mouse and were plotted as group averages ± standard deviations. Open symbols, serum; solid symbols, splenocytes; circles, GST; inverted triangles, A27L, B5R, and D8L; squares, VV-COP.

body weight and general health (unpublished data). As expected, GST-immunized mice exhibited a continuous decline in ear temperature and reached an average temperature below 33°C by day 6 postchallenge, which was statistically significant ($P < 0.009$) (Fig. 4C and G). Interestingly, the only other treatment group for which there was a statistically significant drop in body temperature consisted of mice immunized with the A27L and B5R proteins in MPL-TDM adjuvant ($P < 0.003$,) and this drop was observed only at day 6, not before or after this day. Mice that received the two-protein combination in TiterMax, the triple proteins in MPL-TDM or TiterMax, or the VV-COP scarification maintained their body temperatures after virus challenge, and there was no statistically significant difference between the pre- and postchallenge temperatures on any day. These data imply that recombinant-protein or live-vaccinia virus immunization helps to protect mice from virus-induced adverse changes in body temperature.

For the purpose of assessing the efficacy of recombinant-protein vaccines in preventing severe disease that can result from uncontrolled virus replication and/or dissemination, virus-challenged mice were additionally monitored and scored every day for signs of illness starting on day 4 postchallenge. VV-COP-immunized mice showed no signs of illness (DI, 0) for the duration of the experiment (Fig. 4D and H). In contrast, GST-immunized mice looked slightly ruffled (DI, 1) by day 4 and progressively became very sick between days 6 and 8 (DI, 4 or 5). Mice that received A27L and B5R in either MPL-TDM or TiterMax adjuvant and those that received A27L, D8L, and B5R in TiterMax showed slight ruffling (DI, 1) between days 4 and 7 but did not show any additional signs of illness. In these mice, the slight ruffling disappeared, and the mice appeared normal by day 8. Mice immunized with the triple proteins in MPL-TDM adjuvant appeared slightly sicker (DI, 2) than the other protein-immunized groups by day 6 and showed slower kinetics in returning to normal appearance by day 10. Taken together, the disease index and temperature data suggest that despite a transient but significant loss in body weight, protein immunization can protect mice from disease symptoms that are usually indicative of viral pneumonia, viremia, and systemic spread.

Passive immunotherapy with protein immune serum, not splenocytes, protects mice from lethal vaccinia virus challenge. Previous studies have shown that for mice or macaques vaccinated with live vaccinia virus and depleted of $CD4^+$ or $CD8^+$ T cells, antibody was necessary and sufficient for protection against vaccinia virus- or monkeypox virus-induced disease (3, 10). In addition, passive transfer of vaccinia virus immune sera obtained from vaccinated animals was shown to be protective (3, 10, 30). T cells, however, can provide protection necessary for survival and recovery in the absence of effective antibody responses (3, 56). These studies indicate that both cellular and humoral responses can contribute to optimal protection against vaccinia virus. Because we found that immunization of mice with A27L, D8L, and B5R proteins led to the production of humoral and cellular responses, we were interested in examining the relative contributions of these im-

mune components in protecting mice from lethal challenge. Thus, we performed passive transfer studies with sera and splenocytes isolated from triple-protein-immunized BALB/c mice. None of the naïve mice that received serum or splenocytes from GST-immunized mice survived the challenge (Fig. 5A). Conversely, 10% and 100% of the mice that received splenocytes or serum from triple-protein-immunized mice, respectively, survived the lethal vaccinia virus challenge. All mice that received either splenocytes or serum from VV-COP-vaccinated mice survived the challenge. Progressive weight loss was observed for all the mice until day 8, but mice that received triple-protein serum, VV-COP serum, or VV-COP splenocytes started to make significant recoveries in their weight after day 8 (Fig. 5B). When the VV-COP splenocyte and serum recipient groups were compared, there was no statistically significant difference in weight at any day, although starting on day 8, the mice that had received serum had on average regained more weight. Further, between days 4 and 8, the weight loss experienced by mice treated with protein immune serum was statistically significantly different (P, <0.009) from that for those treated with VV-COP splenocytes or serum. Interestingly, only mice treated with protein immune serum or VV-COP serum or splenocytes maintained their body temperatures, and there was no difference among the groups (Fig. 5C). Compared to mice that were immunized actively (Fig. 4D and H), mice treated passively with protein serum or VV-COP serum or splenocytes appeared more ill up to day 8 to 10, as evidenced by higher disease index scores (Fig. 5D). Taken together, the passive transfer data suggest that for recombinant-protein-immunized mice, antibody may play a major role in the protective immune response, whereas for mice immunized with live virus, both the cellular and humoral responses may contribute to immunity.

DISCUSSION

Immunization with vaccinia virus confers protection against smallpox disease due to the stimulation of a robust, multitarget adaptive immune response that cross-recognizes the highly homologous antigens expressed by variola virus. High-throughput screenings have shown that sera from vaccinia virus-immunized humans, primates, and mice contain antibodies that react with as many as 21 different vaccinia virus proteins (including A27L, D8L, and B5R), which constitute 11% of the total vaccinia virus proteome (7). Given this vast array of immunogenic proteins encoded by vaccinia virus, it has been a difficult task to identify which of those will be as effective as the live virus in providing protection when administered as subunit vaccines. The important roles of IMV in host-to-host transmission and that of EEV in long-range virus dissemination within the infected host (4, 45) have suggested that generating adaptive immune responses that help to prevent both virus infection and dissemination should result in a reduction in the extent of virus-induced disease. In our initial studies, we observed that a vaccine composed only of the IMV proteins A27L, D8L, and H3L in MPL-TDM adjuvant protected only 30% of mice from lethal VV-WR challenge, whereas 100% of the mice were protected when the EEV protein B5R was also included as part of the vaccine (data not shown). We focused on a subunit vaccine formulation composed of A27L, D8L, and B5R for the studies presented in this paper, because vaccination with this three-protein combination provided protection identical to that conferred by a four-protein combination of A27L, D8L, B5R, and H3L (data not shown). Consistent with the protective immunogenicity of these *E. coli*-expressed proteins, the antibodies against them were strongly cross-reactive with the proteins found on the surfaces of vaccinia virions. Most significantly, antibodies to two regions of the B5R glycoprotein (peptides 6 to 8, spanning aa 51 to 90, and peptides 22 to 26, spanning aa 221 to 280) that were identified previously (2) as two major neutralization sites on EEV were also detected in our study. In addition, A27L, D8L, and B5R protein immunization resulted in the generation of high levels of antibodies that target the three functional domains of the viral A27L protein: the heparin binding domain (aa 21 to 33), the fusion domain (aa 29 to 43), and the domain involved in the formation of EEV (aa 1 to 29) (50). The identification of several linear B-cell epitopes that were reactive with the sera of A27L, D8L, and B5R protein-immunized mice is consistent with the assumption that B-cells with receptors that recognize the linear components of the proteins were most likely activated and expanded by vaccination with the *E. coli*-expressed proteins. Future studies will determine whether antibodies to these epitopes play a role in the IMV and/or EEV neutralization observed in the sera of triple-protein-immunized mice. Furthermore, the detection of multiple $CD4^+$ T-cell epitopes within each protein can explain the presence of high levels of protein-specific antibodies, since several studies have demonstrated that the induction of strong antibody responses via vaccination requires $CD4^+$ T-cell help (52, 53).

The in vitro testing of the immunological relevance of immune serum in neutralizing IMV and preventing infections or in aggregating cell-associated enveloped virus and preventing EEV release revealed that the combination of A27L-, D8L-, and B5R-specific antibodies is very effective in inactivating or sequestering vaccinia virus. We believe that these two strategies of antibody targeting of vaccinia virus contribute significantly to the mechanism of survival of protein-immunized mice that were challenged with lethal doses of VV-WR. This view is further supported by data from the passive immunotherapy study in which sera, but not splenocytes, from protein-vaccinated mice provided protection to naïve mice. Although the adoptive transfer of splenocytes was not protective, the possibility of $CD4^+$ T-cell participation in enhancing the recall humoral response of protein-immunized mice cannot be ruled out completely, since peptide-specific memory $CD4^+$ T-cell responses were readily detectable 5 weeks after the last vaccine dose. Despite the significantly low level of virus-reactive antibodies detected in the sera of VV-COP-vaccinated mice 2 weeks postimmunization, the transfer of either serum or splenocytes to naïve mice was found to be protective against lethal VV-WR challenge. Although VV-COP serum collected 2 weeks after immunization exhibited only minimal in vitro IMV neutralization, its effectiveness in neutralizing EEV dissemination was similar to that of serum obtained 4 weeks later. Hence, it is possible that the protection afforded by the passively transferred VV-COP serum may be due mainly to neutralization of EEV rather than of IMV. The protection afforded by adoptively transferred VV-COP immune splenocytes, however, can be attributed to the presence of vaccine-activated $CD4^+$ and/or $CD8^+$ T cells. Consistent with this view, high levels of

peptide-specific $CD4^+$ T cells (specific to the A27L, B5R, and D8L proteins) and $CD8^+$ T cells (specific to the B5R and D8L proteins) were detected by an ELISPOT assay. Not only can these $CD4^+$ T cells potentially provide help for enhanced humoral (53) and $CD8^+$ T-cell (44) responses, but they may also have direct antiviral activity (33). The $CD8^+$ T cells may be involved in direct killing of virus-infected cells and may contribute significantly to survival and recovery from virus challenge in the absence of humoral responses (3, 56). No B5R 14/15- or D8L 12/13-specific $CD8^+$ T cells from A27L-, D8L-, and B5R-vaccinated mice were detected, indicating that immunization with these recombinant proteins does not lead to the generation of significant $CD8^+$ T-cell responses. Taking all of the above observations into consideration, it is possible that the significant weight loss observed in recombinant-protein-immunized mice, but not VV-COP-vaccinated mice, through day 6 post-virus challenge may be due to the absence of memory $CD8^+$ T cells that can complement the humoral response, contribute to the reduction of viral replication, and help prevent the consequences of virus-induced disease.

The production of recombinant proteins in *E. coli* is relatively simple, cost-effective, and scalable to large amounts, and it requires a significantly shorter amount of processing time than protein production in other expression systems—such as yeast, insect, and mammalian cells. These attributes make antigen expression in *E. coli* better suited for the production of large amounts of vaccines quickly in case of an emergency, where there is a shortage in vaccine supply. However, this is dependent on the demonstration that despite the lack of post-translational modification and the potential for the lack of proper folding of proteins produced in *E. coli*, they are as protective as subunit vaccines produced in other expression systems. Our study is important not only in confirming that the A27L, B5R, and D8L proteins produced in *E. coli* are highly immunogenic and elicit strong protective immunity against viral challenge but also in identifying B- and T-cell epitopes that are components of the host immune response. Most importantly, we have detected strong reactivity to the *E. coli*-expressed A27L, D8L, and B5R proteins in human sera from several individuals who received Dryvax vaccination (data not shown). This observation, combined with the detection of $CD4^+$ and $CD8^+$ T-cell responses to A27L and B5R from the peripheral blood mononuclear cells of vaccinees, as reported by Tang et al. (48), suggests that the triple-protein vaccination may also induce protective immunity in humans. Future studies with nonhuman primates, assessing the safety, immunogenicity, and efficacy of vaccination with *E. coli*-expressed A27L, D8L, and B5R proteins, will be important in determining the potential of this protein combination as a safe and effective subunit vaccine. It will also be interesting to make a direct comparison of the protective efficacy of immunization with recombinant A27L, B5R, and D8L proteins versus a polytope vaccine incorporating only the minimal $CD4^+$ and $CD8^+$ T-cell and linear B-cell epitopes of each protein identified in this study.

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ADDENDUM

After the submission of this article, Sakhatskyy et al.(42) reported similar data showing that immunization with *E. coli*-expressed vaccinia virus or variola virus A27L, B5R, and D8L proteins elicits high-titer, cross-reactive, vaccinia virus-neutralizing antibody responses and protects mice against virus challenge.

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